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Two-Laboratory Collaborative Study on Identification of Mycobacteria: Molecular versus Phenotypic Methods

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Previous studies have indicated that the conventional tests used for the identification of mycobacteria may (i) frequently result in erroneous identification and (ii) underestimate the diversity within the genus *Mycobacterium*. To address this issue in a more systematic fashion, a study comparing phenotypic and molecular methods for the identification of mycobacteria was initiated. Focus was given to isolates which were difficult to identify to species level and which yielded inconclusive results by conventional tests performed under day-to-day routine laboratory conditions. Traditional methods included growth rate, colonial morphology, pigmentation, biochemical profiles, and gas-liquid chromatography of short-chain fatty acids. Molecular identification was done by PCR-mediated partial sequence analysis of the gene encoding the 16S rRNA. A total of 34 isolates was included in this study; 13 of the isolates corresponded to established species, and 21 isolates corresponded to previously uncharacterized taxa. For five isolates, phenotypic and molecular analyses gave identical results. For five isolates, minor discrepancies were present; four isolates remained unidentified after biochemical testing. For 20 isolates, major discrepancies between traditional and molecular typing methods were observed. Retrospective analysis of the data revealed that the discrepant results were without exception due to erroneous biochemical test results or interpretations. In particular, phenotypic identification schemes were compromised with regard to the recognition of previously undescribed taxa. We conclude that molecular typing by 16S rRNA sequence determination is not only more rapid (12 to 36 h versus 4 to 8 weeks) but also more accurate than traditional typing.

Mycobacteria are aerobic rod-shaped organisms characterized by being acid fast and having a slow growth rate (31). In addition to those of the *Mycobacterium tuberculosis* complex, other species of mycobacteria are opportunistic pathogens and can pose a serious threat to infected individuals (20, 32). It is important to identify mycobacteria to the species level both to address the clinical significance, e.g., isolation of a nonpathogenic versus pathogenic species, as well as to meet the demands of patient management, since treatment regimens for infections caused by one *Mycobacterium* species are often not effective against another.

Identification of mycobacteria to the species level by conventional biochemical tests has been fraught with a long turnaround time, leading to significant delays in diagnosis. Other methods based on lipid analysis, such as high-performance liquid chromatography, thin-layer chromatography, and gas-liquid chromatography, are cumbersome and expensive and are used in only a very few clinical laboratories (3, 11, 13, 16, 20). Identification by use of nucleic acid probes (Gen-Probe, Inc., San Diego, Calif.) is a rapid and widely used procedure, but it covers only a narrow range of mycobacterial species, and problems concerning specificity and sensitivity have been described (2, 4, 6, 12, 15, 17, 25).

To meet the necessity for more rapid species identification and to improve the accuracy of identification of mycobacteria, methods utilizing the amplification of DNA by PCR coupled with restriction enzyme digestion, hybridization, or nucleic acid sequence determination have been developed (5, 7, 19, 21, 23, 26, 27, 29, 30). The use of 16S rRNA gene sequence determination for the routine identification of mycobacteria has led to the suggestion that the standard biochemical reactions used for the identification of mycobacteria may underestimate the complexity of the genus *Mycobacterium*, as genetically distinct species may exhibit similar or identical patterns in these reactions (10). In this study, we sought to systematically compare phenotypic and molecular methods for the identification of mycobacteria by focusing on isolates which yielded inconclusive biochemical test results and thus posed difficulties for identification to the species level.

MATERIALS AND METHODS

Mycobacterial isolates. Clinical isolates were collected at the Clinical Mycobacteriology Laboratory of the Department of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Foundation, Rochester, Minn. The isolates were grown on Löwenstein-Jensen or 7H10 agar and examined for growth rate, gross and microscopic colony morphology, and pigmentation. Identification to the species level was done by gas-liquid chromatography of short-chain fatty acids (Microbial Identification System; Microbial ID, Newark, Del.) and a battery of biochemical key reactions, including Tween hydrolysis, nitrate reduction, arylsulfatase, urease, tellurite reduction, salt tolerance, and semiquantitative catalase (9, 20). The selection of specific biochemical tests for identification was made on an individual basis after the growth rate, pigment production, and colonial morphologic features were considered. Isolates in this study were identified as "most closely resembling" a particular species.

Isolates were coded (MCRO 1, 2, 5 to 8, 10, 12 to 21, 24 to 30, 32 to 34, 36 to 41, and 45 to 48) and shipped to Hannover for sequence analysis. These isolates could not be definitively identified by the combination of biochemical tests and gas-liquid chromatographic analysis.

Nucleic acid analyses. Identification by 16S rRNA sequence determination was done at the Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover. The methods used have been described previously (10).

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In brief, nucleic acids were extracted by simple mechanical lysis of bacterial cells. PCR was performed in a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM (each) deoxynucleoside triphosphates, 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Überingen, Germany), 30 pmol of primer 264 (5′ TGC ACA CAG GCC ACA AGG GA 3′, corresponding to *Escherichia coli* 16S rRNA from positions 1046 to 1027), and 10 pmol of biotinylated primer 285 (5′ GAG AGT TTG ATC CTG GCT CAG 3′, corresponding to *E. coli* 16S rRNA from positions 9 to 29). The thermal profile involved 39 cycles with a 1-min denaturation step at 94°C and a 3-min annealing and extension step at 68°C.

The biotinylated single-stranded DNA template (8) was prepared by use of Dynabeads M-280–streptavidin (Dynal, Hamburg, Germany) and a Dynal MPC-E magnetic separator essentially as described by the manufacturer. A 20-μl Dynabeads solution (10^8 beads per ml) was used for each PCR reaction. The beads were finally resuspended in 20 μl of H_2O. Sequencing was performed with 2 to 5 μl of the Dynabeads single-stranded DNA solution, 0.5 to 1.0 μCi of [α-^{32}P]dCTP at 3.000 Ci/mmol (Amersham Buchler, Braunschweig, Germany), and Sequenase version 2.0 (U.S. Biochemicals, Bad Homburg, Germany) according to standard procedures (U.S. Biochemicals and Dynal recommendations). Two picomoles of sequencing primer 244 (5′ CCC ACT GCT GCC TCC CGT AG 3′, corresponding to *E. coli* 16S rRNA from positions 361 to 342) per reaction was used for the determination of the nucleic acid sequence of hypervariable region A. Sequencing primer 259 (5′ TTT CAC GAA CAA CGC GAC AA 3′, corresponding to *E. coli* 16S rRNA from positions 609 to 590) was used for the determination of the nucleic acid sequence of hypervariable region B. After electrophoresis, gels were fixed in 10% (vol/vol) acetic acid–12% (vol/vol) methanol, dried, and exposed to X-ray film for 6 to 12 h.

Full 16S rRNA gene sequence determination and phylogenetic analyses were done as described previously (22). For the phylogenetic analysis, regions of alignment uncertainty were omitted. Pairwise distances were calculated by weighting nucleotide differences and insertions-deletions equally (Hamming distances). The phylogenetic trees were constructed by using the neighborliness method.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of isolates MCRO 6, 7, 8, 10, 16, 17, 18, 19, and 24 have been deposited in the EMBL database under accession numbers X93032, X93033, X93034, X93026, X93027, X93028, X93029, X93030, and X93031, respectively.

RESULTS

During a period from 1990 to 1992, a total of 37 isolates which demonstrated some conspicuous attributes in their conventional test results and which were presumptively identified on the basis of a biochemical profile most closely resembling that of a particular species were recovered. The isolates were identified as *M. avium-M. intracellulare* complex ($n = 17$), *M. terrae* ($n = 6$), *M. nonchromogenicum* ($n = 1$), *M. scrofulaceum* ($n = 3$), *M. flavescens* ($n = 1$), *M. fortuitum* ($n = 1$), and *M. asiaticum* ($n = 1$) or were not identified ($n = 4$); for a summary of the biochemical test results, see Table 1. The isolates were coded and submitted for sequence analysis. Thirty-four of the 37 isolates were included in this study; 3 isolates were excluded because of overgrowth by nonmycobacterial contaminants or because they represented a mixture of two strains.

Sequence analysis focused on two regions. These regions, termed regions A and B and corresponding to *E. coli* 16S rRNA positions 129 to 267 and 430 to 500, respectively, have been extensively characterized previously and were found to represent nucleic acid sequence stretches suitable for identification of mycobacteria (10, 21). While determination of the sequence of hypervariable region A is the standard approach for routine identification of mycobacteria by 16S rRNA sequencing, sequence analysis of hypervariable region B is especially suited for making higher-order assignments, in particular for the characterization of isolates that cannot be identified by sequence determination of hypervariable region A and that may thus represent hitherto undescribed taxa.

Following partial sequence analysis, isolates were identified by comparing their sequences with published sequences. In cases in which no match was found, isolates were grouped according to sequence identity, and preliminary phylogenetic assignments were postulated from the inspection of the primary sequence as well as from secondary structure analysis of both region A and region B (sequencing results are given in Fig. 1 and 2 and Table 2). Representative isolates from the groups were chosen for full 16S rRNA gene sequence determination to allow for a detailed phylogenetic analysis (18, 22, 24) of the anonymous isolates (Fig. 3 and 4).

To allow for better understanding, the results have been tabulated (Tables 1, 2, and 3). For some of the strains investigated, the following is a detailed description of the results.

(i) A group of isolates (MCRO 2, 6, 12, 13, 29, 39, and 48) were somewhat heterogeneous by biochemical test results, and isolates were identified as either *M. avium-M. intracellulare* complex or *M. terrae*. By partial sequence analysis of regions A and B, these strains were found to be identical; an insertion of 2 nucleotides within helix 18 allowed for an assignment to the *M. terrae-M. nonchromogenicum* complex (1) (Fig. 2). Strain MCRO 6 was chosen for complete 16S rRNA gene sequence determination, and sequencing revealed that this group of isolates falls within the confines of *M. terrae* and *M. nonchromogenicum* (Fig. 4).

(ii) For three isolates (MCRO 7, 10, and 17) conventionally identified as *M. nonchromogenicum* and *M. scrofulaceum*, sequence determination of 16S rRNA region A and B revealed unique sequences. The sequences obtained suggested that these isolates represent rapid growers; an insertion of 2 nucleotides within helix 10 allowed these unique isolates to be assigned to a subgroup of rapid growers characterized as thermotolerant (24) (Fig. 1). Full 16S rRNA sequence analysis confirmed these findings (Fig. 3). One isolate (MCRO 20) which was identified by biochemical means as *M. flavescens* showed nucleic acid sequences within regions A and B identical to those of MCRO 17.

(iii) A number of unique isolates that either remained unidentified after biochemical analysis (MCRO 18, 45, and 46) or were identified as *M. avium-M. intracellulare* complex (MCRO 19, 33, and 34) or *M. scrofulaceum* (MCRO 8) had unique nucleic acid sequences within region A and were predicted to be closely related to *M. simiae* by analysis of region B. These predictions were tested and verified by full sequence analysis (only the data for MCRO 8 and 19 are given in Fig. 4).

(iv) Eight isolates which were found to correspond to established species by sequence analyses yielded inconclusive results by biochemical tests. These isolates most closely resembled the following species according to the biochemical tests. Two *M.* "*paraffinicum*" isolates were classified as *M. avium-M. intracellulare* complex (MCRO 21 and 38), four *M. celatum* isolates were classified as *M. avium-M. intracellulare* complex (MCRO 25, 26, 27, and 41), one isolate of *M. simiae* was classified as *M. avium-M. intracellulare* complex (MCRO 30), and one isolate of *M. malmoense* was classified as *M. terrae* (MCRO 40).

(v) Two isolates which were presumptively identified by conventional tests as most closely resembling *M. terrae* (MCRO 16 and 24) revealed unique 16S rRNA sequences, although higher-order analysis allowed the assignment of these isolates to the *M. terrae-M. nonchromogenicum* complex.

(vi) Five isolates gave identical results by biochemical and molecular typing methods: three *M. avium-M. intracellulare* isolates (MCRO 32, 36, and 37), one *M. asiaticum* isolate (MCRO 15), and one *M. fortuitum* isolate (MCRO 47).

DISCUSSION

Differentiation of *Mycobacterium* species has traditionally relied upon biochemical test profiles of pure cultures, methodologies which are cumbersome and require 3 to 6 weeks before skilled microbiology technicians can report results. Standard biochemical identification schemes may yield ambig-

TABLE 1. Biochemical test results

Isolate[a]	Result for[b]:								Organism most closely resembled
	Tween hydrolysis	Nitrate	Aryl-sulfatase	Urease	Tellurite reduction	Salt tolerance	Semiquantitative catalase (mm)	GLC[c]	
2	−	−	ND	−	+	−	>45	<0.3	*M. avium-M. intracellulare*
6	−	−	−	−	±	−	>45	<0.3	*M. avium-M. intracellulare*
12	−	−	−	−	±	−	>45	<0.3	*M. avium-M. intracellulare*
13	−	−	ND	−	+	−	>45	<0.3	*M. avium-M. intracellulare*
29	+	−	−	−	±	−	>45	*M. terrae* 0.269	*M. terrae*
39	+	−	−	−	±	−	>45	<0.3	*M. terrae*
48	+	−	ND	−	±	−	>45	ND	*M. terrae*
16	+	+	ND	ND	+	+	>45	<0.3	*M. terrae*
24	+	−	ND	−	+	ND	ND	<0.3	*M. terrae*
7	+	±	−	−	+	±	<45	<0.3	*M. nonchromogenicum*
10	+	−	ND	+	+	−	>45	*M. scrofulaceum* 0.623	*M. scrofulaceum*
17	+	+	ND	+	+	−	>45	*M. avium-M. intracellulare* 0.400	*M. scrofulaceum*
20	+	+	ND	+	−	−	>45	*M. avium-M. intracellulare* 0.400	*M. flavescens*
14	±	−	−	ND	+	−	>45	*M. smegmatis* 0.398	Unidentified
8	+	−	ND	−	±	−	<45	<0.3	*M. scrofulaceum*
18	−	−	ND	−	−	−	<45	<0.3	Unidentified
19	−	−	ND	−	+	−	<45	*M. scrofulaceum* 0.554	*M. avium-M. intracellulare*
33	−	±	ND	ND	−	−	>45	<0.3	*M. avium-M. intracellulare-M. scrofulaceum*
34	+	−	ND	−	±	−	<45	<0.3	*M. avium-M. intracellulare*
45	−	−	ND	−	±	−	0	<0.3	Unidentified
46	ND	−	−	−	−	−	0	<0.3	Unidentified
15	−	−	ND	−	−	−	>45	*M. asiaticum* 0.624	*M. asiaticum*
21	−	−	ND	−	−	−	>45	*M. avium* 0.500	*M. avium-M. intracellulare*
38	−	−	ND	−	−	ND	>45	<0.3	*M. avium-M. intracellulare*
25	−	+	ND	−	+	−	<45	*M. avium* 0.379	*M. avium-M. intracellulare*
26	−	+	+	−	−	−	<45	*M. avium* 0.399	*M. avium-M. intracellulare*
27	−	+	+	−	−	−	<45	*M. avium* 0.398	*M. avium-M. intracellulare*
41	±	+	ND	−	ND	−	<45	*M. avium* 0.300	*M. avium-M. intracellulare*
30	−	+	ND	−	+	−	>45	<0.3	*M. avium-M. intracellulare*
40	+	+	ND	+	−	−	<45	<0.3	*M. terrae*
47	ND	+	−	ND	+	ND	ND	<0.3	*M. fortuitum*
32	−	+	ND	−	+	−	>45	<0.3	*M. avium-M. intracellulare*
36	−	+	−	−	−	−	<45	*M. avium* 0.500	*M. avium-M. intracellulare*
37	−	+	ND	+	+	−	<45	*M. avium* 0.500	*M. avium-M. intracellulare*

[a] The growth rate for MCRO 7, 10, 17, and 20 was >12 days. The niacin test was not performed with MCRO 8, 18, 19, 30, 33, 34, 45, and 46.
[b] −, negative; +, positive; ±, usually positive; ND, not done.
[c] GLC, gas-liquid chromatography. The number corresponds to the similarity index with the Microbial Identification System software.

uous and misleading results, as (i) the tests used may not be highly reproducible, (ii) the phenotype of a species is not an absolute property and may exhibit quite remarkable variability, and (iii) the database of phenotypic characteristics is limited to common species. Because of phenotypic and interassay variability, it is often difficult to decide whether isolates that cannot be assigned to an established species on the basis of biochemical reactions merely reflect phenotypic or interassay variability or represent a previously unknown species. The 16S rRNA sequence is an appealing target for the purpose of genotypic identification (33). It is a stable property, and it contains nucleic acid information allowing the identification of mycobac-

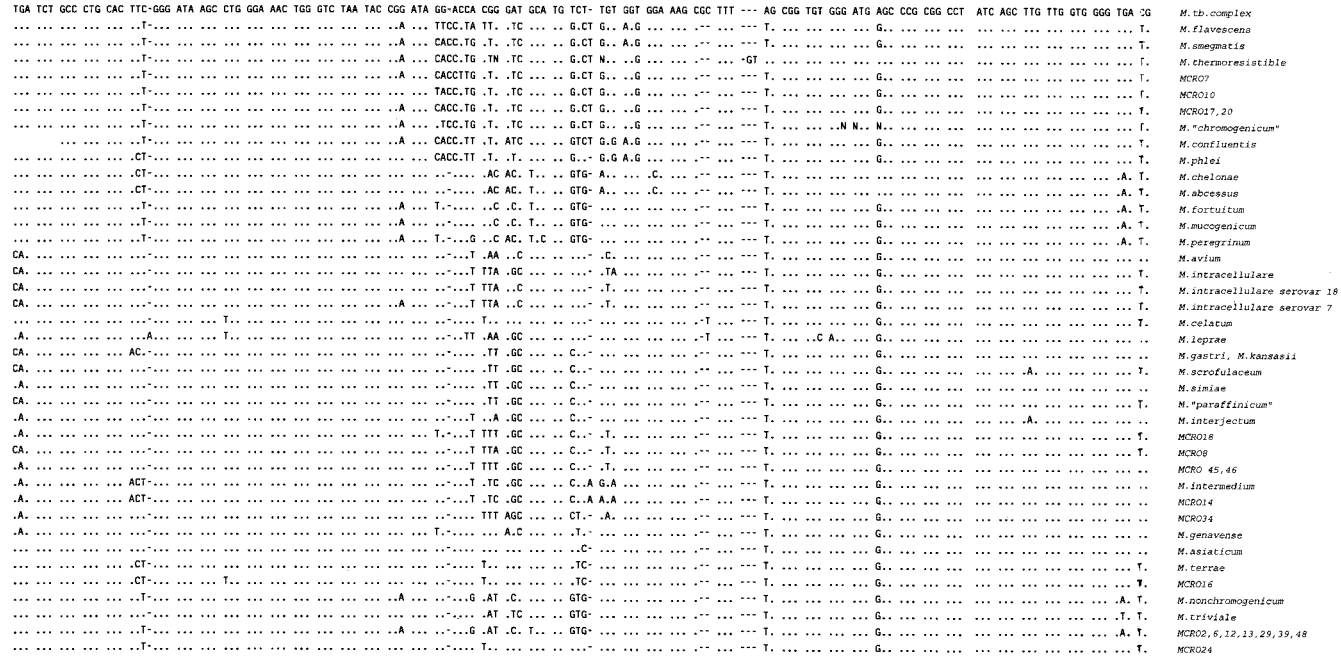

FIG. 1. Alignment of selected mycobacterial 16S rRNA gene sequences within hypervariable region A comprising helix 10. *M. tuberculosis* was used as the reference sequence. Nucleotides different from those of *M. tuberculosis* are indicated; dashes indicate deletions, and dots indicate identity. The first nucleotide corresponds to *E. coli* 16S rRNA position 129; the two nucleotide insertions characterizing a subgroup of rapid growers, provisionally termed thermotolerant, are at positions 186 and 205 of the alignment.

teria at the species level as well as the rapid recognition of previously undescribed mycobacterial pathogens (10, 21).

In this study, we sought to systematically compare traditional biochemical tests with 16S rRNA sequence determination for the purpose of the characterization of isolates which were difficult to identify by conventional tests. At the Mayo Clinic, this would represent approximately 7% of all mycobacteria identified in the clinical laboratory. Specifically, we wanted to tackle the question of whether the biochemical reactions used for the identification of mycobacteria underestimate the complexity of the genus *Mycobacterium* because of the problems of both phenotypic variability and phenotypic homogeneity. For identification by biochemical procedures, a standard battery of tests established for routine identification in a clinical laboratory was used (9, 20). 16S rRNA sequence analysis, which was introduced recently as a routine procedure for the identification of mycobacteria in the clinical laboratory, was performed by concentrating on two previously characterized regions: region A, corresponding to *E. coli* positions 129 to 267, and region B, corresponding to *E. coli* positions 430 to 500. While region A has been used for routine identification, the additional analysis of region B is especially useful for isolates which

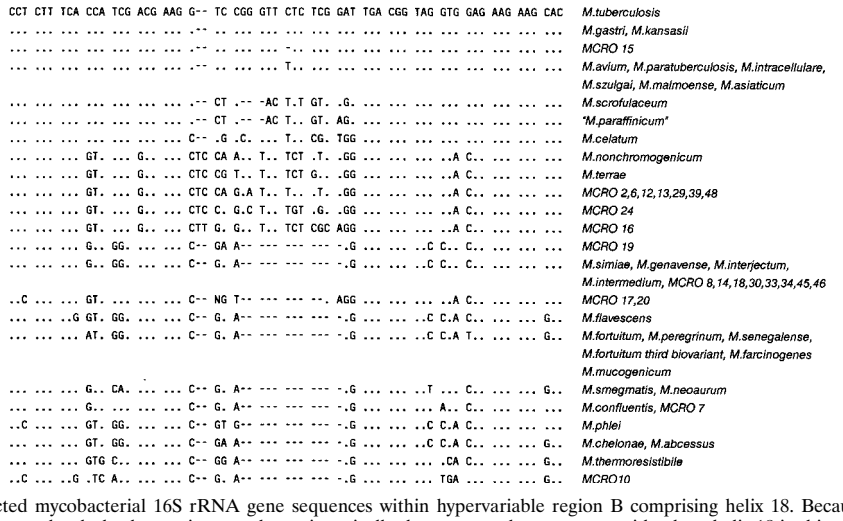

FIG. 2. Alignment of selected mycobacterial 16S rRNA gene sequences within hypervariable region B comprising helix 18. Because of an insertion of several nucleotides at the primary sequence level, slowly growing mycobacteria typically show a secondary structure with a long helix 18 in this region; the additional insertion of 2 nucleotides at positions 456 and 457 characterizes strains of the *M. terrae-M. nonchromogenicum* complex. N, undetermined nucleotide. Other details are given in the legend to Fig. 1.

TABLE 2. 16S rRNA signature sequencing results

Isolate	Result for positions:		Presumptive identification
	120 to 260	430 to 500	
2 6 12 13 29 39 48	Unique and identical sequence	Unique and identical sequence	Related to *M. terrae-M. nonchromogenicum* because of insertion of 2 nucleotides in helix 18
16	Unique sequence	Unique sequence	Related to *M. terrae-M. nonchromogenicum* because of insertion of 2 nucleotides in helix 18
24	Unique sequence	Unique sequence	Related to *M. terrae-M. nonchromogenicum* because of insertion of 2 nucleotides in helix 18
7	Unique sequence	*M. confluentis*	Related to a group of thermotolerant rapid growers, which include, among others, *M. smegmatis*, *M. flavescens*, and *M. confluentis*; this group of organisms shows an insertion of 2 nucleotides in helix 10
10	Unique sequence	Unique sequence	Related to a group of thermotolerant rapid growers which show an insertion of 2 nucleotides in helix 10
17 20	Unique and identical sequence	Unique and identical sequence	Related to a group of thermotolerant rapid growers which show an insertion of 2 nucleotides in helix 10
14	Unique sequence (one nucleotide difference from *M. intermedium*)	*M. simiae*	Slowly growing species closely related to *M. intermedium*
8	Unique sequence	*M. simiae*	Slowly growing species related to *M. simiae*
18	Unique sequence	*M. simiae*	Slowly growing species related to *M. simiae*
19	*M. "paraffinicum"*	*M. simiae*-like	Slowly growing species related to *M. simiae*
45 46	Unique and identical sequence	*M. simiae*	Slowly growing species related to *M. simiae*
33	*M. scrofulaceum*	*M. simiae*	Slowly growing species related to *M. simiae*
34	Unique sequence	*M. simiae*	Slowly growing species related to *M. simiae*
15	*M. asiaticum*	*M. asiaticum*	*M. asiaticum*
21 38	*M. "paraffinicum"*	*M. "paraffinicum"*	*M. "paraffinicum"*
25 26 27 41	*M. celatum*	*M. celatum*	*M. celatum*
30	*M. simiae*	*M. simiae*	*M. simiae*
40	*M. malmoense*	*M. malmoense*	*M. malmoense*
47	*M. fortuitum*	*M. fortuitum*	*M. fortuitum*
32 36 37	*M. intracellulare*	*M. intracellulare*	*M. intracellulare*

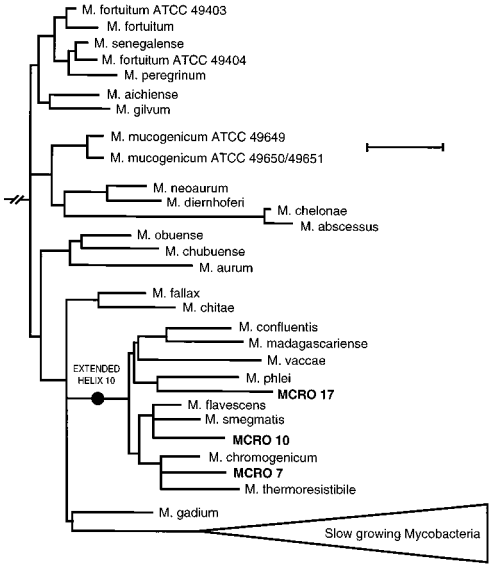

FIG. 3. Phylogenetic tree of the rapidly growing mycobacteria. The tree is based on 16S rRNA gene sequences and illustrates the positions of anonymous MCRO isolates. The tree was rooted by using *Nocardia asteroides* as the outgroup. The bar indicates 10 nucleotide differences.

show unique sequences in region A, possibly indicating previously undescribed taxa (10, 21). We were satisfied to see that the preliminary assignments of anonymous *Mycobacterium* spp. made by partial sequence analysis of regions A and B were confirmed by full 16S rRNA gene sequence determinations in every case.

Two important conclusions emerge from the results of this study.

(i) Variations in phenotypic tests may result in the assignment of one taxon to different species (e.g., MCRO 2, 6, 12, 13, 17, 20, 39, and 48) as well as in false identification results (e.g., MCRO 21, 30, and 40). Accordingly, discrepant results by

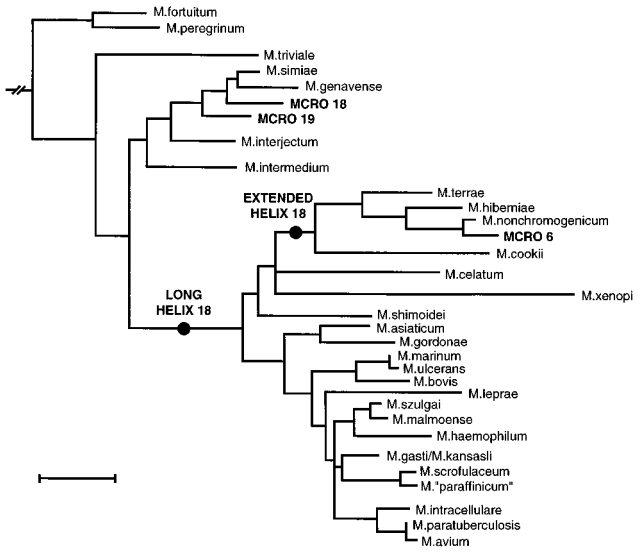

FIG. 4. Phylogenetic tree of the slowly growing mycobacteria. The tree is based on 16S rRNA gene sequences and illustrates the positions of anonymous MCRO isolates. The tree was rooted by using *Nocardia asteroides* as the outgroup. The bar indicates 10 nucleotide differences.

TABLE 3. Biochemical and molecular identification results

Isolate	Biochemical test result	16S rRNA sequencing result
2	*M. avium-M. intracellulare*	Unique and identical group of microorganisms related to *M. terrae-M. nonchromogenicum*
6	*M. avium-M. intracellulare*	
12	*M. avium-M. intracellulare*	
13	*M. avium-M. intracellulare*	
29	*M. terrae*	
39	*M. terrae*	
48	*M. terrae*	
16	*M. terrae*	Unique isolate related to *M. terrae-M. nonchromogenicum*
24	*M. terrae*	Unique isolate related to *M. terrae-M. nonchromogenicum*
7	*M. nonchromogenicum*	Unique isolate related to a group of thermotolerant rapid growers
10	*M. scrofulaceum*	Unique isolate related to a group of thermotolerant rapid growers
17	*M. scrofulaceum*	Unique and identical group of microorganisms related to a group of thermotolerant rapid growers
20	*M. flavescens*	
14	Unidentified	Closely related to *M. intermedium*
8	*M. scrofulaceum*	Unique isolate related to *M. simiae*
18	Unidentified	Unique isolate related to *M. simiae*
19	*M. avium-M. intracellulare*	Unique isolate related to *M. simiae*
45	Unidentified	Unique and identical group of microorganisms related to *M. simiae*
46	Unidentified	
33	*M. avium-M. intracellulare*	Unique isolate related to *M. simiae*
34	*M. avium-M. intracellulare*	Unique isolate related to *M. simiae*
21	*M. avium-M. intracellulare*	*M. "paraffinicum"*
38		
25	*M. avium-M. intracellulare*	*M. celatum*
26		
27		
41		
30	*M. avium-M. intracellulare*	*M. simiae*
40	*M. terrae*	*M. malmoense*
15	*M. asiaticum*	*M. asiaticum*
47	*M. fortuitum*	*M. fortuitum*
32	*M. avium-M. intracellulare*	*M. intracellulare*
36		
37		

conventional and molecular typing methods could be attributed to erroneous interpretations or results of biochemical tests.

(ii) Phenotypic identification schemes are especially compromised by hitherto unrecognized taxa (e.g., MCRO 7, 8, 10, 17, 19, 33, and 34), which are often erroneously misassigned to established species.

Our results indicate that traditional biochemical identification schemes may not be appropriate for the recognition of previously uncharacterized pathogens. This phenomenon is probably due to (i) interassay and phenotypic variability, (ii) phenotypic homogeneity, with its concomitant lack of differential characteristics, and (iii) the bias of traditional identification schemes towards established taxa.

As was demonstrated by our results, the inherent bias of phenotypic identification schemes results in underestimation of the microbial diversity within the genus *Mycobacterium*. In taxonomic terms, we deal with three steadily expanding subgroups within the mycobacteria which are characterized by specific molecular signatures.

(i) The first is a group of species which exhibit slow growth and yet show the molecular signature of rapidly growing species, i.e., a short helix 18. These species have a nucleic acid sequence in hypervariable region B that is identical to that of *M. simiae*, e.g., *M. genavense*, *M. intermedium*, *M. interjectum*, MCRO 8, MCRO 18, and MCRO 33. As has been pointed out previously, "the most striking thing about *M. simiae* is the difficulty of precise information.. . . This may be due to extreme test variability for the species, but our past experience suggests that an equally plausible explanation is the possibility that the species may still be a mixture of two or more specific species or subspecies" (9).

(ii) The second is a group of slowly growing species which are characterized by a long helix 18 which is extended by 2 nucleotides (1). This group comprises *M. terrae*, *M. nonchromogenicum*, *M. hiberniae*, MCRO 6, MCRO 16, and MCRO 24.

(iii) A distinct branch of rapidly growing mycobacteria shows an insertion of 2 nucleotides in helix 10, resulting in a helix containing 11 bp, while all other mycobacteria have a helix that contains 10 bp. This group, initially characterized as thermotolerant (24), includes *M. smegmatis*, *M. flavescens*, *M. confluentis*, *M. phlei*, *M. thermoresistibile*, *M. madagascariense*, *M. vaccae*, MCRO 7, MCRO 10, and MCRO 17.

We hesitate to address the taxonomic implications of the findings reported in this study, i.e., the level of separation from established taxa at a species or subspecies level. Although we here refrain from the formal description of novel species of mycobacteria, it is clear by the number of nucleic acid sequence differences found within the 16S rRNA molecule that a significant fraction of the isolates investigated in this study represent novel, previously undescribed species. Our results demonstrate that phenotypic identification schemes have blurred our recognition of diversity within the mycobacteria. Such problems as phenotypic and interassay variability as well as phenotypic homogeneity and concomitant lack of differential characteristics in the standard tests used for identification contributed to this phenomenon.

16S rRNA sequence determination has some distinct and important advantages compared with other molecular identification strategies. It is a universally applicable procedure which allows not only proper identification of isolates, i.e., assignment to already established taxa, but also rapid recognition and classification of previously undescribed pathogens. Given the ease of nucleic acid sequence determination and a steadily increasing database which currently includes more than 2,500 bacterial species (14, 28), we expect that the approach of 16S rRNA gene sequence determination will become a standard means for identification of microorganisms in the clinical laboratory, especially microorganisms which are difficult to identify by the use of more traditional techniques.

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